Cancer Chemother Pharmacol (1987) 20: 193–197

Cancer Chemotherapy and Pharmacology

Concurrent allopurinol and 5-fluorouracil: 5-fluoro-2'-deoxyuridylate formation and thymidylate synthase inhibition in rat colon carcinoma and in regenerating rat liver*

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Summary. The formation of FdUMP and the inhibition of TS were studied in a subcutaneously growing transplantable rat colon carcinoma and in regenerating rat liver following bolus administration of 5-FU, with or without HPP pretreatment.

In tumor, peak levels of FdUMP at 30 min following bolus 5-FU, 100 mg/kg, averaged 4931 ± 587 pmol/g. Pretreatment with HPP, 50 mg/kg, 24 h and 1 h before 5-FU, reduced the peak FdUMP level to 2085 ± 387 pmol/g. The inhibition of TS by 5-FU treatment was greater than 95% by 30 min, and after 48 h residual enzyme inhibition averaged 40%. No effect on TS inhibition by 5-FU treatment could be observed as a result of HPP pretreatment. The levels of TS_{tot} increased linearly after 5-FU treatment and doubled within 48 h.

In regenerating rat liver, neither FdUMP levels nor TS inhibition, studied at 1 h after bolus 5-FU, were affected by HPP pretreatment.

Introduction

A major growth-inhibitory effect of 5-FU is associated with formation of the anabolite FdUMP. FdUMP in the presence of the cofactor CH_2FH_4 binds covalently to TS, thus inactivating the enzyme and thereby blocking thymidylate production for DNA synthesis [18, 24]. There are also cytotoxic effects caused by the ribonucleotide FUTP, which is incorporated into and interferes with the function of RNA [5, 30]. This leads to inhibition of ribosomal maturation and alteration in post-transcriptional modification of transfer RNA [7, 17].

There are three metabolic pathways that convert 5-FU to nucleotides. The thymidine phosphorylase/thymidine kinase pathway may be of minor importance because of low tissue levels of deoxyribose donors [21]. The uridine phosphorylase/uridine kinase pathway and the phosphoribosyl transferase pathway activate 5-FU to FUMP, the precursor of FdUMP and FUTP [21]. Should the uridine phosphorylase/uridine kinase pathway predominate in neoplastic tissue, and the phosphoribosyl transferase pathway be more important in normal tissue, the therapeutic index of 5-FU could be increased by selectively blocking the phosphoribosyl transferase pathway by the use of HPP.

HPP is an analogue of hypoxanthine in which the 7-nitrogen and 8-carbon of the purine ring are formed in reversed position as compared with the naturally occurring purines. HPP inhibits the enzyme xanthine oxidase and thereby blocks the convertion of hypoxanthine to uric acid. However, HPP is also converted by xanthine oxidase to 1-oxipurinol, which is subsequently phosphorylated by hypoxanthine-guanine phosphoribosyl transferase to 1-oxypurinol-5'-monophosphate, a potent inhibitor of orotidylate decarboxylase. The blockage of this enzymatic step in the de novo purine synthesis pathway may result in increased levels of orotate that could compete with 5-FU for the available PRPP pool for activation by orotate phosphoribosyl transferase [8, 10, 12, 26] resulting in decreased formation of FdUMP. Furthermore, and perhaps more important, the inhibition of xanthine oxidase leads to purine accumulation resulting in prolonged depletion of PRPP [15]. HPP pretreatment allows doubling of the maximal tolerated dose of intravenous 5-FU. However, HPP pretreatment has not been shown to alter the response rate to 5-FU alone in patients with colorectal adenocarcinoma [9, 16, 27]. Leissner and Gustavsson, though, reported complete responses in patients with multiple bladder tumors after intravesical high-dose 5-FU instillations combined with oral allopurinol, which eliminated the development of cystitis, which attends intravesical 5-FU instillations [19, 29.] Clark and Slevin reported that six patients with mucositis due to 5-FU treatment against colerectal cancer, experienced a decrease in mucositis after treatment with allopurinol mouth washes [6].

Our present goal was to study the effects of HPP pretreatment on 5-FU-mediated TS inhibition in a solid rat tumor model and in regenerating rat liver following bolus 5-FU. For this purpose ligand binding methods for analysis of the intracellular concentration of FdUMP and TS were used [20, 28].

* This study was supported by grants from the Swedish Cancer Society, The Medical Society of Göteborg, the University of Göteborg, the Wellcome Foundation, and NCI Grant 39629.

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Abbreviations: BSA, bovine serum albumin; DMH, dimethylhydrazine; 5-FU, 5-fluorouracil; HPP, allopurinol (4-hydroxypyrazolopyrimidine); CH_2FH_4, 5,10-methylenepteroylmonoglutamic acid; FUMP, 5-fluorouridine-5'-monophosphate; FUTP, 5-fluorouridine-5'-triphosphate; FdUMP, 5-fluoro-2'-deoxyuridylate; TS, thymidylate synthase (EC 2.1.1.45, dTMP synthase); TS_f, free, non-FdUMP-bound TS; TS_b, ternary complex-bound TS; TS_{tot}, total TS $TS_f + TS_b$; PRPP, phosphoribosylpyrophosphate

Materials and methods

Reagents. L. casei TS was purchased from the New England Enzyme Center, Boston, Mass. CH_2FH_4 was freshly prepared for each experiment by addition of 4.0 μmol *dl*-L-tetrahydropteroylmonoglutamate (Sigma) to 50 μl 1 *M* ascorbate (pH 6.5), 2.5 μl 37% formaldehyde (w/v) and 1.95 ml of a buffer consisting of 50 m*M* potassium phosphate (pH 7.2), 20 m*M* 2-mercaptoethanol and 2% (w/v) BSA [28]. 5-FU was purchased from Roche AB, Sweden, and allopurinol (HPP) was a gift from The Wellcome Foundation Ltd, Stockholm, Sweden. FdUMP was purchased from Sigma, and 6-[^{3}H]FdUMP (18 Ci/mmol) was obtained from Moravek Biochemicals, Brea, California. The stock solution of charcoal was prepared by adding BSA and T-70 Dextran (Pharmacia) to 10% neutral charcoal (Sigma) as previously described [20]. This solution was diluted 1:3 in 0.2 *M* HCl before use. All other chemicals used were of analytical grade and commercially available. FdUMP dilutions were made in 10 m*M* phosphate, and TS dilutions were made in the buffer used for tissue homogenization (see below).

Tumor model. Male and female Wistar rats were used at 15–20 weeks of age (weight 100–180 g). They were maintained on chow and water ad libitum. The tumor was a DMH-induced colon carcinoma obtained from the Wallenberg Laboratory, Lund, Sweden and showed intermediate grades of differentiation. It was passaged in the host of origin by i. p. injection of cell suspension and was studied at transplant generation 65. Fifty-four rats were inoculated with 1 ml tumor cell suspension subcutaneously in the lumbar region. The experiments were performed 2 weeks later when the tumors were 1.4±0.6 g (mean ± SD) in size. Half of the animals were injected i.p. with bolus 5-FU, 100 mg/kg, alone and the other half were pretreated with i.p. injections of HPP, 50 mg/kg, 24 h and 1 h prior to 5-FU injection. Three rats were killed at each time point studied. Tumors were immediately frozen in liquid N_2 and stored at −80° C after excision prior to tissue processing.

Regenerating rat liver model. At time zero, HPP 50 mg/kg was administered i.p. to 15 rats. At 1h, standard liver resection was performed [11]. At 23 h another 50 mg/kg HPP was given. At 24 h 100 mg/kg 5-FU was given i.p., and 1 h later the rats were killed. The regenerating livers were removed and handled as described (Fig. 1). Another 15 rats were treated in the same way except that no HPP was given.

Preparation of homogenates. The tissues were thawed at 4° C and placed in a fourfold excess of a 0.2 *M* Tris HCl buffer (pH 7.4) containing 20 m*M* 2-mercaptoethanol, 15 m*M* cytidylate and 100 m*M* NaF, disrupted by use of a ground glass homogenizer, and sonicated 3 × 20 s (100 W).

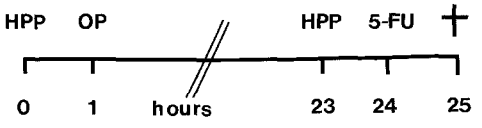

Fig. 1. Experimental setup for determination of HPP pretreatment effects on 5-FU-mediated TS inhibition in regenerating rat liver. *OP,* operation (standard liver resection)

Aliquots were taken for nucleotide extraction, and the remainder of the crude sonicates were centrifuged (10000 g for 10 min) and stored at 4° C for cytosol preparation for TS assay.

Nucleotide extraction. This was done according to Nazar et al. [22]. First 300–500 μl crude sonicate was placed in a tenfold excess of 1 *M* acetic acid, freeze-thawed three times and centrifuged. The supernatant was collected and the procedure was then repeated twice on the pellet. The three supernatants were pooled and lyophilized to dryness. Separation of dUMP from FdUMP was done using DEAE-cellulose columns as previously described [23].

Biochemical assays. FdUMP and TS as free [^{3}H]FdUMP-titrable binding sites (TS_f) or as endogenous ternary complex-bound enzyme (TS_b) were measured by the methods developed by Moran et al. [20] and Spears et al. [28].

TS assay. TS_f was measured by adding 50 μl cytosol to 6 pmol 6-[^{3}H]FdUMP plus 50 nmol CH_2FH_4 in a total volume of 175 μl. After 20 min at 30° C, 1.0 ml 3% ice-cold charcoal was added with vortexing. The tubes were centrifuged at 4000 *g* for 20 min at 4° C, and 600 μl of the supernatant was removed for scintillation counting. Corrections were made for exchange labeling of cytosolic TS_b enzyme with [^{3}H]FdUMP. TS_{tot} was determined after dissociation of the ternary complex at pH 8 for 4 h followed by addition of CH_2FH_4 and excess [^{3}H]FdUMP as in the TS assay [20]. Due to the very high levels of FdUMP, cytosols were treated with an equal volume of ice-cold 10% neutral charcoal prior to TS assay in order to remove nucleotides [3].

FdUMP assay. The lyophilized nucleotide extract was dissolved in 5 m*M* potassium phosphate. Then 25 μl of this was added to 0.3 pmol [^{3}H]FdUMP, 0.15 pmol TS and 50 nmol CH_2FH_4 in a total volume of 125 μl. After 2 h incubation at 30°, 1.0 ml ice-cold acidified 3% charcoal was added for isolation of charcoal-resistant radioactivity.

Results

FdUMP kinetics and TS inhibition in tumor

The intracellular pharmacokinetics of FdUMP formation and TS inhibition in tumor during a 48-h period following i.p. bolus 5-FU (100 mg/kg), with or without i.p. pretreatment with 50 mg HPP/kg (given 24 h and 1 h before 5-FU administration), are presented in Figs. 2 and 3. The highest levels of FdUMP, 4931±557 pmol/g, were observed within 30 min after bolus 5-FU alone. In the HPP-pretreated tumors the peak FdUMP values were found at 60 min and reached only 2085±307 pmol/g. The elimination of FdUMP appeared to be exponential, and after 22 h no free FdUMP was detectable (Fig. 2). At time points beyond 2 h, no differences in the FdUMP values could be observed. The kinetics of TS inhibition and recovery after bolus 5-FU, 100 mg/kg, are outlined in Fig. 3. The pretreatment TS value was 19.2±2.0 pmol/g. There was a rapid decrease of free TS enzyme after bolus 5-FU, and at 15 min, the first time point studied, there was less than 0.5 pmol/g TS_f. The inhibition was almost complete up to 4 h. At 24 h TS_f reached pretreatment values despite the continued presence of FdUMP bound to TS. This was explained by

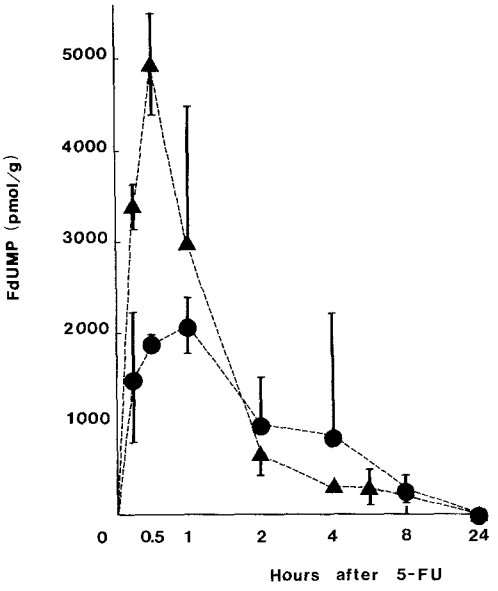

Fig. 2. Intracellular concentration of FdUMP in rat colon adenocarcinoma as a function of time after bolus 5-FU, 100 mg/kg, with and without HPP pretreatment, 50 mg/kg, 24 h and 1 h before 5-FU. *Symbols:* FdUMP in tumor after 5-FU alone (▲) and in combination with HPP (●)

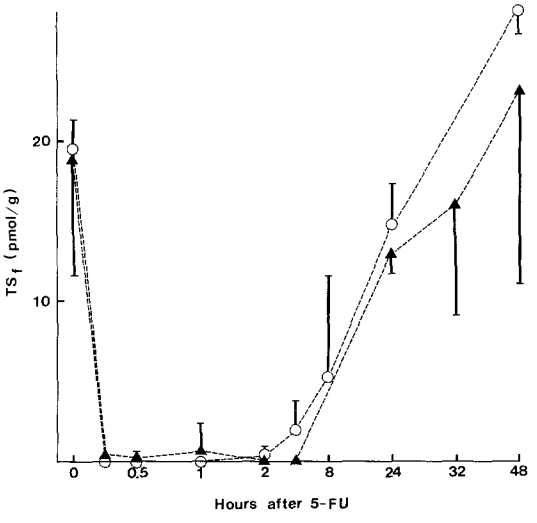

Fig. 3. In vivo kinetics of TS_f in rat colon adenocarcinoma following bolus 5-FU, 100 mg/kg, with (○) and without (▲) HPP pretreatment. Each point represents the mean ± SD in three rats

an increase in TS_{tot} to approximately 40 pmol/g during the 48-h observation period (Fig. 4). HPP did not change the kinetics of TS inhibition. Thus, a 50% reduction of the FdUMP concentration did not result in impaired TS inhibition.

FdUMP levels and TS inhibition in regenerating rat liver

The levels of FdUMP and TS 1 h after bolus 5-FU are presented in Table 1. FdUMP values averaged 163 ± 131 and 193 ± 78 pmol/g (mean ± SD) with and without HPP pretreatment respectively, i.e., approximately one-tenth of the FdUMP levels in tumor 1 h after 5-FU. The TS_{tot} levels averaged 6.4 ± 2.8 pmol/g, approximately one-third the pre-

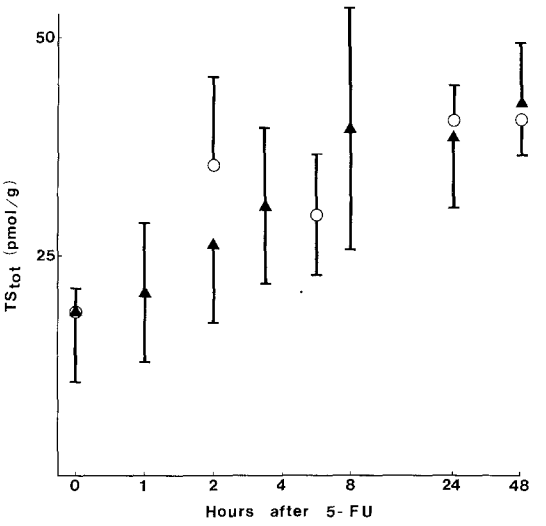

Fig. 4. TS_{tot} as a function of time after bolus 5-FU, 100 mg/kg, with (○) and without (▲) HPP pretreatment. Each point represents the mean ± SD in 3 rats

treatment level in tumor. TS_f averaged 1.5 ± 1.0 and 1.4 ± 0.8 pmol/g with and without HPP pretreatment respectively. Thus, the percentage TS inhibition at 1 h following bolus 5-FU was approximately 77%. No differences in neither FdUMP concentration nor TS inhibition could be demonstrated as a result of the HPP pretreatment.

Discussion

We have recently reported that administration of 5-FU to rats following resection of the liver, results in significant FdUMP formation and TS inhibition in the regenerating liver [4]. We postulated that this could become a clinical problem when patients subjected to liver resection or suffering from liver cirrhosis were treated with 5-FU. In order to find out whether the regenerating liver could be protected against 5-FU cytotoxicity by HPP, the levels of FdUMP and TS inhibition were studied 1 h after bolus 5-FU with or without HPP pretreatment. Apparently, neither formation of FdUMP nor TS inhibition was affected by the HPP pretreatment. There are several explanations for this: The uridine phosphorylase/uridine kinase pathway for FdUMP formation may predominate in the regenerating liver. Furthermore, it is known, that HPP reduces the clearance of 5-FU [15], resulting in prolongation of the 5-FU half-life. Although it has not been shown, this may be a result of reduction of the hepatic metabolism of 5-FU. Houghton and Houghton reported that co-administration of HPP actually increased the formation of fluoro-

Table 1. Parameters of TS inhibition in regenerating rat liver 1 h after i.p. administration of 5-FU, 100 mg/kg, with or without HPP pretreatment

	5-FU	5-FU + HPP
FdUMP	193 ± 78	163 ± 131
TS_{tot}	6.4 ± 2.8	
TS_f	1.4 ± 0.8	1.5 ± 1.1

nucleotides in 5-FU-sensitive xenografts of human colonic carcinomas in mice up to 7 days following bolus 5-FU [12]. They concluded that the HPP-induced increase in purines and the subsequent increse of ribose-l-phosphate from the breakdown of inosine monophosphate could improve the conversion of 5-FU to its nucleosides.

In contrast to the findings concerning regenerating liver, HPP pretreatment did have effects on FdUMP fromation in our tumor model. Peak FdUMP levels were reduced from 5000 to 2000 pmol/g if HPP was given before the bolus 5-FU injection, indicating that the phosphoribosyl transferase pathway plays an important role in the anabolism of 5-FU to FUMP. However, 2000 pmol FdUMP per gram tissue was still more than enough to provide complete TS inhibition, and the inhibition of TS was almost complete from the first time point studied (15 min) up to 4 h, and then TS_f slowly recovered. It is possible that when lower doses of 5-FU are used, thus generating lower levels of FdUMP, the HPP-mediated reduction of FdUMP formation may result in decreased TS inhibition. While investigating the pathways of 5-FU metabolism in five human colerectal adenocarcinoma xenografts, Houghton and Houghton found that the tumors could be divided into two groups according to the way in which 5-FU is metabolized to ribonucleotides. In tumors with high uridine phosphorylase/phosphoribosyl transferase ratios and high ribose-1-phosphate/PRPP ratios, HPP and hypoxanthine did not reduce the formation of 5-FU ribonucleotides. In tumors with low ratios of those enzymes and substrates, HPP and hypoxanthine did reduce the formation of 5-FU ribonucleotides due to depletion of PRPP [13].

In tumor, TS_{tot} values increased in a linear fashion following bolus 5-FU, and were doubled after 48 h independent of HPP pretreatment. A similar increase in TS_{tot} was observed by Spears et al. in all of three investigated murine colon adenocarcinomas that were resistant to 5-FU [28]. The 5-FU-induced increase in TS has not yet been completely elucidated, but could theoretically be explained by a synchronization of the cells into the S phase or new synthesis of the enzyme induced by TS inhibition [1], initiated perhaps by gene amplification [2].

Although this study has failed to demonstrate an effect of HPP pretreatment on the 5-FU-mediated inhibition of TS in regenerating liver and tumor, it is possible that there are major effects of HPP on the formation of FUTP and the incorporation of fluoronucleotides into RNA. The mechanism of 5-FU toxicity to the GI tract in mice has been reported to be correlated with its incorporation into RNA [14]. It has also been reported that incorporation of 5-FU into RNA of bone marrow and GI tissue in mice were reduced by 50% in the presence of HPP [25].

At 4–8h, free TS appeared to return in spite of substantial amounts of FdUMP still present in the cytosols. This could theoretically be explained by dUMP pool expansion or depletion of the folate cofactor.

In conclusion, this study has demonstrated that HPP pretreatment does not reduce the formation of FdUMP and the inhibition of TS in regenerating rat liver following bolus 5-FU injection. In this DMH-induced colon carcinoma, however, the formation of FdUMP was reduced as a consequence of HPP pretreatment, but the TS inhibition was not affected. This may indicate that once the desired high percentage TS inhibition is obtained, further increases in 5-FU dose have little effect on the inhibition of TS, because the disappearance of FdUMP is faster than the dissociation of the ternary complex.

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Received November 12, 1986/Accepted July 13, 1987